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| TRANSMITTAL LETTER TO THE UNITED STATES<br>DESIGNATED/ELECTED OFFICE (DO/EO/US)<br>CONCERNING A FILING UNDER 35 U.S.C. 371   |   | ATTORNEY'S DOCKET NUMBER<br><b>TEI-120</b>      |
|  |   | U.S. APPLICATION NO. (if known, see 37 CFR 1.5) |
| INTERNATIONAL APPLICATION NO.<br><b>PCT/JP00/04146</b>   | INTERNATIONAL FILING DATE<br><b>October 5, 2000</b> | PRIORITY DATE CLAIMED<br><b>October 5, 1999</b> |
| <b>TITLE OF INVENTION</b><br><b>METHODS FOR INHIBITING OSTEOCLAST FORMATION OR METHODS FOR INHIBITING BONE RESORPTION</b>  |   |   |
| <b>APPLICANT(S) FOR DO/EO/US</b><br><b>Yoshiaki AZUMA, Tomohiro OHTA, Keiji KOMORIYA, Akira KUDO, Sunao TAKSHITA, Keisuke KAJI</b>   |   |   |
| <p>Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:</p> <ol style="list-style-type: none"> <li>1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.</li> <li>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.</li> <li>3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(I).</li> <li>4. <input type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</li> <li>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <ul style="list-style-type: none"> <li>a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau</li> <li>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li> </ul> </li> <li>6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</li> <li>7. <input checked="" type="checkbox"/> Amendment to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)). <ul style="list-style-type: none"> <li>a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input type="checkbox"/> have been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendment has NOT expired.</li> <li>d. <input checked="" type="checkbox"/> have not been made and will not be made.</li> </ul> </li> <li>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</li> <li>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</li> <li>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</li> </ol> <p>Items 11 to 16 below concern either document(s) or information included:</p> <ol style="list-style-type: none"> <li>11. <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</li> <li>12. <input checked="" type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</li> <li>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <ul style="list-style-type: none"> <li><input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</li> </ul> </li> <li>14. <input type="checkbox"/> A substitute specification.</li> <li>15. <input type="checkbox"/> A change of power of attorney and/or address letter.</li> <li>16. <input type="checkbox"/> Other items or information.</li> </ol> |   |   |

|   |  |                                     |                       |                 |      |
|---|--|-------------------------------------|-----------------------|-----------------|------|
| U.S. APPLICATION NO. (If known, see 37 CFR 1.5)   | INTERNATIONAL APPLICATION NO<br>PCT/JP00/04146 | ATTORNEY'S DOCKET NUMBER<br>TEI-120 |                       |                 |      |
| 17. <input checked="" type="checkbox"/> The following fees are submitted:   |  | CALCULATIONS PTO USE ONLY           |                       |                 |      |
| <b>Basic National Fee (37 CFR 1.49(a)(1)-(5):</b><br>Search Report has been prepared by the EPO or JPO.....   |  | \$860.00                            |                       |                 |      |
| International preliminary examination fee paid to USPTO (37 CFR 1.482)  |  |                                     |                       |                 |      |
| No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).....  |  |                                     |                       |                 |      |
| Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....   |  |                                     |                       |                 |      |
| International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....  |  |                                     |                       |                 |      |
| <b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>   |  | <b>\$ 860.00</b>                    |                       |                 |      |
| Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.49(e)).  |  | \$                                  |                       |                 |      |
| Claims  | Number Filled                                  | Number Extra                        | Rate                  |                 |      |
| Total   | Claims   | 6-20 =                              | 0                     | X \$18          | \$ 0 |
| Independent   | Claims   | 2-3 =                               | 0                     | X \$78          | \$ 0 |
| Multiple dependent claim(s) (if applicable)   |  |                                     | + \$260               | \$ 0            |      |
| <b>TOTAL OF ABOVE CALCULATIONS</b>  |  |                                     | =                     | <b>\$860.00</b> |      |
| Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28)   |  |                                     |                       | \$              |      |
| <b>SUBTOTAL</b>   |  |                                     | =                     | <b>\$860.00</b> |      |
| Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.49(f)).   |  |                                     | + \$                  | \$              |      |
| <b>TOTAL NATIONAL FEE</b>   |  |                                     | =                     | <b>\$860.00</b> |      |
| Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate sheet (37 CFR 3.28, 3.31). \$40.00 per property  |  |                                     | + \$ 0                | \$ 0            |      |
| <b>TOTAL FEES ENCLOSED</b>  |  |                                     | =                     | <b>\$860.00</b> |      |
|   |  |                                     | Amount to be refunded | \$              |      |
|   |  |                                     | Charged               | \$860.00        |      |
| a. <input type="checkbox"/> A check in the amount of \$ _____ to cover the above fees is enclosed.<br>b. <input checked="" type="checkbox"/> Please charge my Deposit Account No <u>18-0013</u> in the amount of <u>\$860.00</u> to cover the above fees. A duplicate of this sheet is enclosed.<br>c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>18-0013</u> . A duplicate copy of this sheet is enclosed. |  |                                     |                       |                 |      |
| NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.   |  |                                     |                       |                 |      |
| SEND ALL CORRESPONDENCE TO:<br><br>David K. Benson<br>Rader, Fishman & Grauer, L.P.P.C.<br>1233 20 <sup>th</sup> Street, N.W.<br>Suite 501<br>Washington, DC 20036<br>202-955-3750  |  |                                     |                       |                 |      |
| <br><b>DAVID K. BENSON</b>   |  |                                     |                       |                 |      |
| REGISTRATION NUMBER 42,314  |  |                                     |                       |                 |      |
| Dated: May 23, 2001   |  |                                     |                       |                 |      |

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the U.S. Nationalization

Application of PCT/JP00/04146

Yoshiaki AZUMA et al.

Attn: Application Branch

Application No. To Be Assigned

Filed: May 22, 2001

For: METHODS FOR INHIBITING OSTEOCLAST FORMATION OR METHODS FOR  
INHIBITING BONE RESORPTION

PRELIMINARY AMENDMENT

Commissioner of Patents  
Washington, DC 20231

Sir:

Prior to the initial examination, please amend the above-identified application as follows:

IN THE CLAIMS:

Please amend claim 5 and add new claims 7 and 8 as follows:

5. (Amended) The method for inhibiting bone resorption as described in claim 3 wherein  
said co-culture comprises an inducing factor of osteoclast formation and/or bone resorption in the  
co-culture medium.

7. The method for inhibiting bone resorption as described in claim 4 wherein said co-  
culture comprises an inducing factor of osteoclast formation and/or bone resorption in the co-  
culture medium.

8. The method for inhibiting osteoclast formation or the method for inhibiting bone  
resorption according to claim 7 wherein said inducing factor comprises at least one factor

selected from the group consisting of macrophage colony-stimulating factor, osteoclast forming factor, tumor necrosis factor, interleukin-1, interleukin-3, interleukin-6, interleukin-11, interleukin-15, interleukin-17, prostaglandins, parathyroid hormone, parathyroid hormone-related peptide, granulocyte macrophage colony-stimulating factor, active vitamin D and its derivatives.

**R E M A R K S**

This preliminary amendment is requested to change all multiple dependency claims to single dependency claims to reduce the initial filing fee. No new matter has been added. Entry of this amendment is requested.

Respectfully submitted,



David K. Benson  
Reg. No. 42,314

Date: May 23, 2001

**RADER, FISHMAN & GRAUER, PLLC**  
The Lion Building  
1233 20<sup>th</sup> Street, N.W., Suite 501  
Washington, D.C. 20036  
Tel: (202) 955-3750  
Fax: (202) 955-3751  
Customer No. 23353

JC18 Rec'd PCT/PTO 23 MAY 2001

**MARKED-UP VERSION TO SHOW CHANGES MADE****IN THE CLAIMS:**

Please amend claim 5 as follows:

5. (Amended) The method for inhibiting bone resorption as described in claim 3 [or 4] wherein said co-culture comprises an inducing factor of osteoclast formation and/or bone resorption in the co-culture medium.

## DESCRIPTION

METHOD FOR INHIBITING OSTEOCLAST FORMATION OR  
METHOD FOR INHIBITING BONE RESORPTION

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## Technical Field

The present invention relates to a method for inhibiting osteoclast formation, or a method for inhibiting bone resorption.

More particularly this invention relates to a method for inhibiting osteoclast formation or a method for inhibiting bone resorption by ultrasound (which may be called as "US" hereinafter) treatment.

## Background Art

A known and conventional method for inhibiting osteoclast formation or for inhibiting bone resorption includes a method consisting of adding a bisphosphonate to a co-culture system [Bilezikian, JP., Raisz, LG., and Rodan, GA. eds., "Principles of bone biology," Academic Press, New York (1996)]. Clinically, a medicine effective in inhibiting osteoclast formation or inhibiting bone resorption has been used for treating of a bone disease caused by abnormal bone resorption due to osteoclast [Bilezikian, JP., Raisz, LG., and Rodan, GA. eds., "Principles of bone biology," Academic Press, New York (1996)].

In the method for adding or administering of a medicine or pharmaceuticals, however, the medicine or pharmaceuticals are taken into body or cells directly, and thus it is not free from the risk of causing some side-effects. Therefore, there is a demand for a method whereby it is possible to inhibit osteoclast formation or bone resorption, while being less likely to cause side-effect than does the conventional method.

In view of this, the present inventors had tried hard to find such a method as mentioned above, that is, a method whereby it is possible to inhibit osteoclast formation or bone resorption, in spite of being less likely to cause side-effect than does the conventional method, and had observed that stimulation with ultrasound, which is physical and non invasive, can effectively inhibit osteoclast formation or bone resorption, and achieved this

invention.

#### Disclosure of the Invention

The present invention provides a method for inhibiting osteoclast formation or a method for inhibiting bone resorption which comprises exposing cells to ultrasound in the culture containing an osteoclast precursor and an inducing factor of osteoclast formation and /or bone resorption, or a method for inhibiting osteoclast formation or a method for inhibiting bone resorption which comprises exposing cells to ultrasound in the co-culture containing an osteoclast precursor and a supporting cell for osteoclastogenesis.

#### Brief Description of the Drawings

Figure 1 shows the number of osteoclasts over time in an experiment where the development of mouse osteoclast precursors into osteoclasts is stimulated by sRANKL.

Figure 2 shows the number of osteoclasts obtained when mouse osteoclast precursors have been co-cultivated with mouse bone marrow cells for 96 hours.

Figure 3 shows the number of osteoclasts obtained when mouse osteoclast precursors have been co-cultivated with mouse bone marrow cells for 120 hours.

Figure 4 shows the area of resorption pits by osteoclasts which have been derived from mouse osteoclast precursors co-cultivated with mouse bone marrow cells.

Note that, for all the relevant Figures, \* represents the test result is significantly different from the result of control group at  $p<0.05$  based on the Student's t-test, while \*\*\* represents the same at  $p<0.001$ .

#### Best Mode for Carrying Out the Invention

The present invention is described below in detail.

The osteoclast precursor used in this invention is the precursor that develops into osteoclasts in the presence of an inducing factor added to the culture medium, or in the presence of supporting cells added to the culture

medium which have been known from the effect to other osteoclast precursors to have an activity to support osteoclast formation. As such osteoclast precursors, bone marrow-derived precursors, spleen-derived precursors and blood monocyte-derived precursors may be mentioned. 5 Those precursor cells may be obtained from the bone marrow, spleen or peripheral blood of humans, mice, etc., and they can be cultivated through passage on a culture medium specially prepared for the emphasized growth of such osteoclast precursors.

The inducing factor which stimulates osteoclast precursors to turn them 10 into osteoclasts is at least one chosen from the group comprising a macrophage colony stimulating factor (to be referred to as "M-CSF" hereinafter), osteoclast forming factor (RANKL/TRANCE/ODF/OPGL, or to be referred to as "RANKL" hereinafter), tumor necrosis factor (the same as above "TNF"), interleukin 4 (IL-4), and vascular endothelial cell growth 15 factor (VEGF). Those factors may be used alone or in combination. RANKL is preferably used, or an artificially synthesized soluble RANKL is most preferred when used at a concentration of 1 to 250 ng/ml. Addition of the inducing factor into the culture medium should be occurred during a period immediately after the inoculation of osteoclast precursors onto the medium 20 till osteoclast formation.

Besides the osteoclast precursors, the co-culture system may include at least one chosen from the group comprising osteoblasts, stroma cells, fibroblasts, T-cells and B-cells. A single kind or several kinds of those cells may be included.

25 Moreover, the co-culture system preferably includes an inducing factor. The inducing factor may include, in addition to M-CSF, RANKL, TNF, IL-4 and VEGF mentioned above, interleukin-1 (to be referred to as "IL-1" hereinafter), interleukin-3 (the same as above "IL-3"), interleukin-6 (the same as above "IL-6"), interleukin-11 (the same as above "IL-11"), interleukin-15 (the same as above "IL-15"), interleukin-17 (the same as above "IL-17"), prostaglandins (the same as above "PG") including prostaglandin E2, 30 parathyroid hormone (the same as above "PTH"), parathyroid hormone-related peptide (the same as above "PTHrP"), granulocyte/macrophage colony stimulating factor (the same as above

"GM-CSF"), and activated vitamin Ds including 1  $\alpha$ ,25-dihydroxyvitamin D3 (the same as above "VD"). They may be used alone or in combination.

Of all the inducing factors cited above, RANKL is preferred to be used. This is because RANKL is a factor essential for the osteoclast formation, and 5 plays an important role in bone metabolism/maintenance [Takahashi, N., et al., Biochem Biophys Res Comm 256: 449-455 (1999)]. RANKL is mainly present on the surface of cells known to support the osteoclast formation, such as osteoblasts, stroma cells, fibroblasts, etc., and induces osteoclast formation by binding with receptors (RANK/TRANCER) of the osteoclast 10 precursors [Takahashi, N., et al., Biochem Biophys Res Comm 256: 449-455 (1999)]. Because RANKL hardly exist as a soluble factor in the body, the co-culture system for the osteoclast formation may be thought as a system resembling an *in-situ* environment involved in the bone metabolism. RANKL plays an important role in the metabolism of a normal bone, but it is 15 also deeply involved in the development of bone-related diseases. For example, in postmenopausal osteoporosis, the number of osteoclasts and their bone resorbing activity are increased, and the bone mass decreased. It is thought that RANKL is involved in the growth of osteoblasts [Kong, YY. et al., Nature 397:315-323 (1999)]. It has been demonstrated that a biological 20 molecule called osteoclast formation inhibiting factor (to be referred to as "OPG/OCIF" herinafter) that interferes with the mutual binding of RANKL molecules not only promotes the osteoclast formation stimulated by RANKL, but also, in animal models having the diseases resulting from the enhanced 25 osteoclast formation or enhanced activity of osteoclasts with bone-related diseases such as hypercalcemia, osteoporosis, etc., inhibits the osteoclast formation , and also improves the symptoms such as bone mass reduction and increase of calcium level in blood, by suppressing the bone absorbing activity. [Lacey DL., et al., Cell 93: 165-176 (1998)]. If it becomes possible to 30 develop a method by which to inhibit the osteoclast formation stimulated by RANKL or induced via a certain agent stimulated by RANKL, as a therapeutic means to recover the reduced bone mass or bone disruption in bone-related diseases resulting from the enhanced bone resorption such as osteoporosis, chronic rheumatoid arthritis, etc.: the reduced bone mass or the loosening after arthroplasty; or the disruption of a bone as a result of cancer

metastasized to the bone, etc., the method will be prospective as a therapy to treat bone-related diseases.

The co-culture system used in this invention is a culture system in which osteoclast precursors and at least one type of cells which support osteoclast formation are cultivated in the same medium. The cell to support osteoclast formation is at least one cell selected from the group consisting of osteoblasts, stroma cells, fibroblasts, T-cells and B-cells. They may be used alone or in combination. Of those cells, stroma cells are preferably used, and bone marrow-derived stroma cells are more preferred. The most preferred combination consists of bone-marrow derived osteoclast precursors and bone marrow-derived stroma cells. The bone marrow-derived stroma cells may include mouse bone marrow-derived stroma cells such as ST-2, TSB-13-9, etc.

The cell line ST-2 is available from American Type Culture Collection, and it can be cultivated and maintained on the culture medium that will be detailed later, to be ready for experimentation.

The medium used in this invention to cultivate osteoclast precursors may include alpha MEM medium containing 8 to 1000 ng/ml of M-CSF and supplemented with 5 - 15% fetal bovine serum. Particularly, the alpha MEM medium containing 100 ng/ml of M-CSF and supplemented with 10% fetal bovine serum is preferred.

The medium used for the co-culture system may include alpha MEM medium supplemented with an inducing factor described above, and 5 - 15% fetal bovine serum. Particularly, alpha MEM medium supplemented with 1  $\alpha$ ,25-dihydroxy vitamin D3 having a concentration of  $1 \times 10^{-10}$ M to  $1 \times 10^{-7}$  M, and 10% fetal bovine serum is preferred. The inducing factor may be added to the medium during a period between a time when osteoclast precursors and osteoclast formation supporting cells such as bone marrow-derived stroma cells are inoculated concomitantly into the medium and a time when osteoclasts are formed.

The ultrasound to be used in this invention includes ultrasound with an output too low to generate heat. More specifically, the ultrasound is a low-output pulsed ultrasound consisting of a series of bursts of which the wave component in a burst has a frequency of 1.3 to 2 MHz; the repetition

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frequency of bursts is 100 to 1000 KHz; the width of a burst is 10 to 2000  $\mu$ sec; and the output intensity is 100 mW/cm<sup>2</sup> or less. Exposure of the ultrasound is achieved, to cite an example for illustration, as follows: an ultrasound generator provided by Exogen is so adjusted as to give low-output ultrasound pulses lasting 20 minutes in total for a day; the generator is so positioned with respect to the culture as to expose the wave to the bottom of the culture well; and the exposure continues for three to five days. The preferred properties of the ultrasound exposure, for example, are as follows; the width of a burst was 200  $\mu$ sec, the frequency was 1.5 MHz, the repetition frequency of bursts was 1 kHz and the output intensity was 30 mW/cm<sup>2</sup>.

The ultrasound generator to be used in this invention is not limited to any specific one, but, if clinically used, what is preferred is, for example, a generator enabling basic, non-invasive therapy as disclosed in US patent (No. 4,530,360) conferred to Duarte, which is capable of applying pulsed ultrasound percutaneously from a point close to the site to be treated. The preferred ultrasound emitted by such a generator may consist of a series of bursts of which the wave component in a burst has a frequency of 1.3 to 2 MHz; the repetition frequency of bursts is 100 to 1,000 Hz; the width of a burst is 10 to 2,000  $\mu$ sec; and the output intensity is 100 mW/cm<sup>2</sup> or less. The duration of exposure is preferably shorter than 20 minutes for a day. Or, an ultrasound-based therapeutic system incorporating a body applicator unit communicable with a remote control unit as disclosed in US Patent (No. 5,003,965) conferred to Talish et al. may be used. The ultrasound in this case is a low-output pulsed ultrasound with a frequency of 1.3 to 2 MHz and an output intensity of 1 to 50 mW/cm<sup>2</sup>.

According to the method of this invention for inhibiting the osteoclast formation or bone resorption, it will be possible to expose ultrasound onto a lesion where bone mass reduction or bone disruption occurs as a result of the enhanced osteoclast formation or enhanced bone resorption, and, if it is clinically applied, to recover the reduced bone mass or bone disruption in bone-related diseases such as osteoporosis, chronic rheumatoid arthritis, etc., to fix aseptic loosening after arthroplasty, and to treat the bone disruption due to a tumor metastasized to a bone. Moreover, the method of this invention for the inhibiting osteoclast formation or bone resorption is

comparatively free from the risk of causing side-effect as compared with the conventional therapy based on the administration of medicines.

### Examples

5 The present invention will be described below by means of examples.

#### Examples 1·1 to 1·4, and Comparative Examples 1·1 to 1·4

10 Mouse bone marrow-derived osteoclast precursors (to be referred to as "MDBM" hereinafter) were obtained by extracting the bone marrow of femur and tibia of mice, separating monocytes according to convention, inoculating the monocytes on dishes to give a concentration of  $4 \times 10^6$  cells/10 cm<sup>2</sup>-dish, and selectively cultivating adhesive cells in the presence of 100 ng/ml of M-CSF for three days. The culture medium was an alpha MEM medium containing 100 ng/ml of M-CSF and supplemented with 10% fetal bovine serum. MDMB was inoculated into a 6-well plate 2 ml for each well to give 15 a concentration of  $1 \times 10^5$  cells/well. To each well, soluble RANKL (to be referred to as "sRANKL" hereinafter) was added to give a concentration of 50 ng/ml. sRANKL was obtained from Repro-Tech. For the experiment, following groups were prepared.

Comparative Example 1·1: control group cultivated for 72 hours.

20 Example 1·1: ultrasound exposure group cultivated for 72 hours. For this group, ultrasound was exposed from the bottom of each well for 20 minutes daily for two days successively.

Comparative Example 1·2: control group cultivated for 96 hours.

Example 1·2: ultrasound exposure group cultivated for 96 hours. 25 For this group, ultrasound was exposed from the bottom of each well for 20 minutes daily for three days successively.

Comparative Example 1·3: control group cultivated for 108 hours.

Example 1·3: ultrasound exposure group cultivated for 108 hours. 30 For this group, ultrasound was exposed from the bottom of each well for 20 minutes daily for four days successively.

Comparative Example 1·4: control group cultivated for 144 hours.

Example 1·4: ultrasound exposure group cultivated for 144 hours. For this group, ultrasound was exposed from the bottom of each well for 20 minutes daily for five days successively.

For both the Comparative Examples and Examples, sRANKL was dissolved in sterilized distilled water to give a concentration of 10  $\mu$ g/ml; and the resulting solution was added so as to be diluted 200 times to give a concentration of 50 ng/ml. On the third day, the media of both the Comparative Examples and Examples were replaced with fresh 10% FBS-aMEM containing 50 ng/ml of sRANKL.

Exposure of ultrasound was not applied to the Comparative Examples 1-1 to 1-4. For Examples 1-1 to 1-3, a low output ultrasound was applied from the bottom of each well for 20 minutes daily using an ultrasound generating unit provided by Exogen which had been modified to be adapted for cell culture. Example 1-1 received ultrasound exposure for two days; Example 1-2 for three days; Example 1-3 for four days and Example 1-4 for five days successively. As to the output properties of the ultrasound applied, the width of a burst was 200  $\mu$ sec, the frequency was 1.5 MHz, repetition frequency was 1 kHz and the output was 30 mW/cm<sup>2</sup>.

Removal of the culture medium took place 72 hours after the onset of cultivation for Comparative Example 1-1 and Example 1-1, 96 hours after for Comparative Example 1-2 and Example 1-2, 108 hours after for Comparative Example 1-3 and Example 1-3, and 144 hours after for Comparative Example 1-4 and Example 1-4. The cells were fixed with phosphate-buffered formalin, stained with tartaric-acid-resistive acidic phosphatase (TRAP) according to convention for identification of osteoclasts, and placed under an optical microscope, and the number of osteoclasts was determined on the premise that a TRAP-positive polynucleated cell having ten or more nuclei is an osteoclast.

The results are shown in Figure 1. As is obvious from the Figure, the numbers of osteoclasts in Examples 1-2 to 1-4 are less than those of Comparative Examples 1-2 to 1-4.

30 Examples 2-1 to 2-2 and Comparative Examples 2-1 to 2-2

MDBMs were obtained by extracting the bone marrow of femur and tibia of mice, separating monocytes therefrom according to convention, inoculating the monocytes on dishes to give a concentration of  $4 \times 10^6$  cells/10 cm-dish, and selectively cultivating adhesive cells in the presence of 100

ng/ml of M-CSF for three days. The culture medium was an alpha MEM medium containing 100 ng/ml of M-CSF and supplemented with 10% fetal bovine serum. The cell line TSB-13·9 derived from mouse bone marrow-derived stroma cells is an established cell line prepared from cells obtained from the bone marrow of SV-40-large-T-antigen transgenic mice. The cells of TSB-13·9 were inoculated on petri dishes to give a concentration of  $2 \times 10^5$  cells/10 cm-dish, and cultivated for three days. The medium was an alpha MEM medium supplemented with 10% fetal bovine serum.

Cells of MDBM and TSB-13·19 were simultaneously inoculated into a 6-well plate 2 ml for each well to give concentrations of  $1 \times 10^5$  and  $1 \times 10^6$  cells/well, respectively. To each well,  $1 \times 10^{-8}$  M  $1\alpha,25$ -dihydroxyvitamin D3 was added. For the experiment, following groups were prepared.

Comparative Example 2-1: control group cultivated for 96 hours.

Example 2-1: ultrasound exposure group cultivated for 96 hours. For this group, ultrasound was exposed from the bottom of each well for 20 minutes daily for three days successively.

Comparative Example 2-2: control group cultivated for 120 hours.

Example 2-2: ultrasound exposure group cultivated for 120 hours. For this group, ultrasound was exposed from the bottom of each well for 20 minutes daily for four days successively.

For both the Comparative Examples and Examples,  $1\alpha,25$ -dihydroxyvitamin D3 was dissolved in ethanol to give a concentration of  $1 \times 10^{-5}$  M; and the resulting solution was added so as to be diluted 1,000 times to give a concentration of  $1 \times 10^{-8}$  M. On the third day, the media of both the Comparative Examples and Examples were replaced with fresh 10% FBS- $\alpha$ MEM containing  $1 \times 10^{-8}$  M  $1\alpha,25$ -dihydroxyvitamin D3.

Exposure of ultrasound was not applied to Comparative Examples 1-1 and 1-2. For Examples 1-1 and 1-2, low output ultrasound was applied from the bottom of each well for 20 minutes daily using an ultrasound generating unit provided by Exogen which had been modified to be adapted for cell culture. Example 1-1 received the ultrasound exposure for three days successively, and Example 1-2 for four days. The ultrasound consisted of the output properties, the width of a burst was 200  $\mu$ sec, the frequency was 1.5 MHz, the repetition frequency of bursts was 1 kHz and the output

intensity was 30 mW/cm<sup>2</sup>.

Removal of the medium took place 96 hours after the onset of cultivation for Comparative Example 1-1 and Example 1-1, and 120 hours for Comparative Example 1-2 and Example 1-2. The cells were fixed with 10% phosphate-buffered formalin, stained with tartaric acid resistive, acid phosphatase (TRAP) according to convention for identification of osteoclasts, and placed under an optical microscope, and the number of osteoclasts was determined on the premise that a TRAP-positive polynucleated cell having ten or more nuclei is an osteoclast. The results are shown in Figure 1.

As is obvious from Figures 2 and 3, the numbers of osteoclasts in Examples 1-1 and 1-2 are less than those of Comparative Examples 1-1 and 1-2.

Examples 3-1 and Comparative Example 3-1

MDBMs prepared as in Example 1 were inoculated onto a 24-well plate 1 ml for each well to give a concentration of  $2 \times 10^4$  cells/well. To each well of the 24-well plate, a hydroxyapatite-coated disc (Osteologic, Millennium Biologix, Inc.) had been placed in advance to receive the cells to grow them thereupon. sRANKL was added to each well to give a concentration of 50 ng/ml. sRANKL had been obtained from Pepro-Tech.

For the experiment, following groups were prepared.

Comparative Example 3-1: control group cultivated for 120 hours.

Example 3-1: ultrasound exposure group cultivated for 120 hours. For this group, ultrasound was exposed from the bottom of each well for 20 minutes daily for four days successively.

For both the Comparative Examples and Examples, sRANKL was dissolved in sterilized distilled water to give a concentration of 10  $\mu$ g/ml; and the resulting solution was added so as to be diluted 200 times to give a concentration of 50 ng/ml. On the third day, the media of both the Comparative Examples and Examples were replaced with fresh 10% FBS- $\alpha$  MEM containing 50 ng/ml of sRANKL.

Exposure of ultrasound was not applied to Comparative Example 3-1. For Example 3-1, a low output ultrasound was applied from the bottom of each well for 20 minutes daily for four days successively using a ultrasound generating unit provided by Exogen which had been modified to be adapted

for cell culture. The ultrasound consisted of the output properties, the width of a burst was 200  $\mu$ sec, the frequency was 1.5 MHz, the repetition frequency of bursts was 1 kHz and the output intensity was 30 mW/cm<sup>2</sup>.

5 Removal of the medium took place 120 hours after the onset of cultivation for both Comparative Example 3-1 and Example 3-1, and the hydroxyapatite-coated discs were removed. The disc was washed with distilled water, and placed under an optical microscope, and absorption of the hydroxyapatite mass by osteoclasts was checked. A picture of a part of the disc (whose area is 50% or more of the total area) was fed through an image 10 processor into a computer, and the area of disc undergoing resorption was determined by means of an image analysis application. The resorbed area relative to the total was determined and the results are shown in Figure 3.

15 As is obvious from Figure. 3, the relative area of disc subjected to osteoclastic resorption is smaller in Example 3-1 than in Comparative Example 3-1.

#### Example 4-1 and Comparative Example 4-1

20 MDBM and the cell line TSB-13-9 derived from mouse bone-marrow derived stroma cells were prepared as in Example 2. The medium was an alpha MEM medium supplemented with 10% fetal bovine serum .

25 MDBM and TSB-13-19 were inoculated at the same time into a 24-well plate to give concentrations of  $2 \times 10^4$  and  $1 \times 10^5$  cells/well, respectively. The volume the culture medium was arranged as 1 ml finally. To each well of the 24-well plate, a hydroxyapatite-coated disc (Osteologic, Millennium Biologix, Inc.) had been placed in advance to receive the cells to grow them thereupon.  $1 \alpha,25$ -dihydroxyvitamin D3 was added to each well to give a concentration of  $1 \times 10^{-8}$  M.

For the experiment, following groups were prepared.

Comparative Example 4-1: control group cultivated for 120 hours.

30 Example 4-1: ultrasound exposure group cultivated for 120 hours. For this group, ultrasound was exposed from the bottom of each well for 20 minutes daily for four days successively.

For both the Comparative Example and Example,  $1\alpha,25$ -dihydroxyvitamin D3 was dissolved in ethanol to give a concentration

of  $1 \times 10^{-5}$  M; and the resulting solution was added so as to be diluted 1,000 times to give a concentration of  $1 \times 10^{-8}$  M. On the third day, the media of both the Comparative Example and Example were replaced with fresh 10% FBS- $\alpha$ MEM containing  $1 \times 10^{-8}$  M  $1\alpha,25$ -dihydroxyvitamin D3.

5 Exposure of ultrasound was not applied to Comparative Example 4-1. For Example 4-1, low output ultrasound was applied from the bottom of each well for 20 minutes daily for four days successively using an ultrasound generating unit provided by Exogen which had been modified to be adapted for cell culture. The ultrasound consisted of the output properties, the width 10 of a burst was 200  $\mu$ sec, the frequency was 1.5 MHz, the repetition frequency of bursts was 1 kHz and the output intensity was 30 mW/cm<sup>2</sup>.

15 Removal of the medium took place 120 hours after the onset of cultivation for both Comparative Example 4-1 and Example 4-1. The disc was washed with distilled water, and placed under an optical microscope, and resorption of the hydroxyapatite mass by osteoclasts was checked. A 20 picture of a part of the disc (whose area is 50% or more of the total area) was fed through an image processor into a computer, and the area of disc undergoing absorption was determined by means of an image analysis application. The resorbing area relative to the total was determined and the results are shown in Figure 4.

As is obvious from Figure 4, the relative area of disc subjected to osteoclastic resorption is smaller in Example 4-1 than in Comparative Example 4-1.

25 The present invention provides a method for inhibiting osteoclast formation or a method for inhibiting bone resorption, characterized in using the exposure of ultrasound. A clinical application to the medical treatment for bone-related deceases, such as a bone mass reduction or a bone disruption by osteoplosis and chronic rheumatoid arthritis, a bone destruction of aseotic loosening with joint arthroplasty just after the replacement with an 30 artificial joint, by ultrasound exposure, which is physical and non-invasive, to a lesion where bone mass reduction or bone destruction occurs as a result of the enhanced osteoclast formation or enhanced bone resorption, without some side-effects, becomes possible. Moreover, the clinical application of the method of this invention for inhibiting the osteoclast formation or bone

resorption will be promising in future, because the risk of causing side-effect is smaller in this method as compared with the conventional therapy based on the administration of medicines.

CLAIMS

1. A method for inhibiting osteoclast formation or a method for inhibiting bone resorption which comprises exposing cells to ultrasound in the culture 5 containing an osteoclast precursor and an inducing factor of osteoclast formation and /or bone resorption.

2. The method for inhibiting osteoclast formation or the method for inhibiting bone resorption according to claim 1 wherein said inducing factor comprises at least one factor selected from the group consisting of 10 macrophage colony-stimulating factor, osteoclast differentiating factor, tumor necrosis factor, interleukin-4, and vascular endothelial cell growth factor.

3. A method for inhibiting osteoclast formation or a method for inhibiting bone resorption which comprises exposing cells to ultrasound in the co-culture containing an osteoclast precursor and an supporting cell for osteogenesis. 15

4. The method for inhibiting osteoclast formation or the method for inhibiting bone resorption according to claim 3 wherein said co-culture system comprises at least one cell seledcted from the group consisting of osteoblast, stroma cell, fibroblast, T-lymphocyte and B-lymphocyte.

5. The method for inhibiting osteoclast formation or the method for inhibiting bone resorption as described in claim 3 or 4 wherein said co-culture 20 comprises an inducing factor of osteoclast formation and/or bone resorption in the co-culture medium.

6. The method for inhibiting osteoclast formation or the method for inhibiting bone resorption according to claim 5 wherein said inducing factor 25 comprises at least one factor selected from the group consisting of macrophage colony-stimulating factor, osteoclast forming factor, tumor necrosis factor, interleukin-1, interleukin-3, interleukin-6, interleukin-11, interleukin-15, interleukin-17, prostaglandins, parathyroid hormone, parathyroid hormone-related peptide, granulocyte macrophage colony 30 -stimulating factor, active vitamin D and its derivatives.

## ABSTRACT

A method for inhibiting osteoclast formation or a method for inhibiting bone resorption which comprises exposing cells to ultrasound in the culture containing an osteoclast precursor and an inducing factor of osteoclast formation and /or bone resorption. Or, a method for inhibiting osteoclast formation or a method for inhibiting bone resorption which comprises exposing cells to ultrasound in the co-culture containing an osteoclast precursor and a supporting cell for osteoclastogenesis.

10

Figure 1

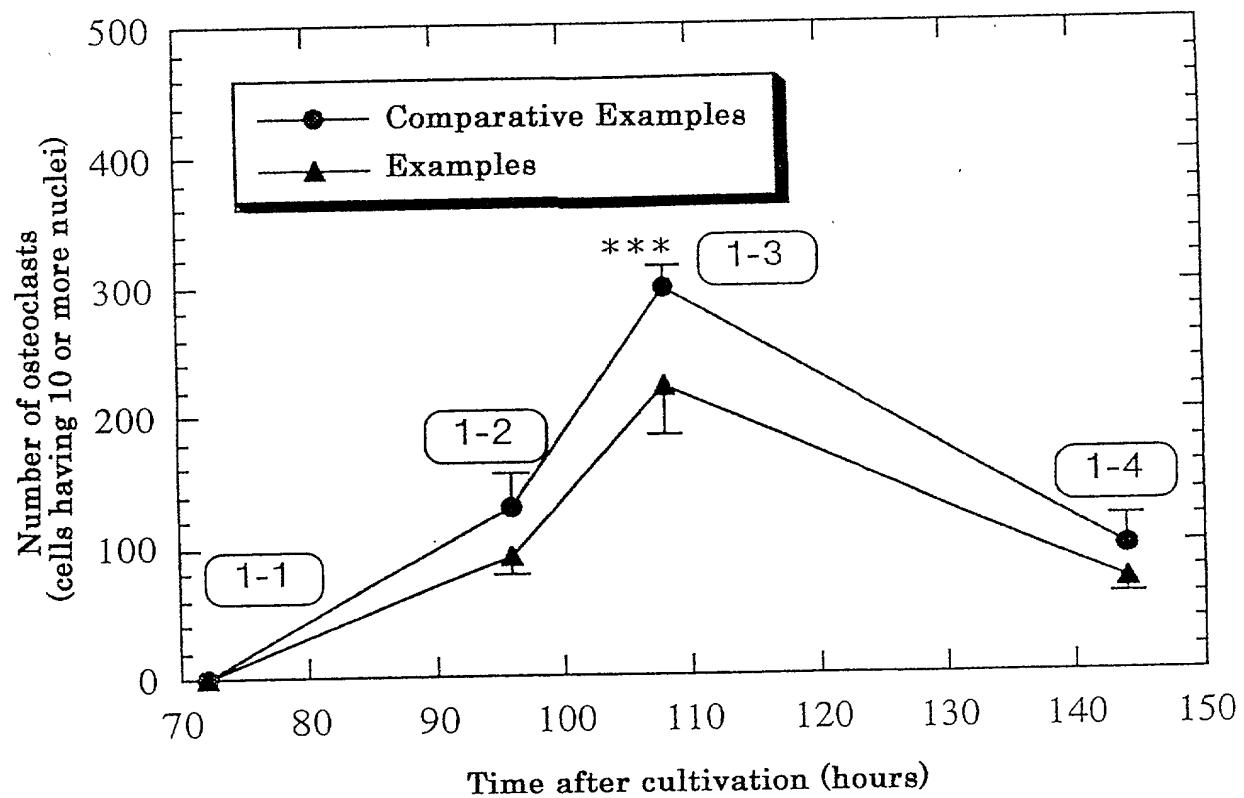


Figure 2

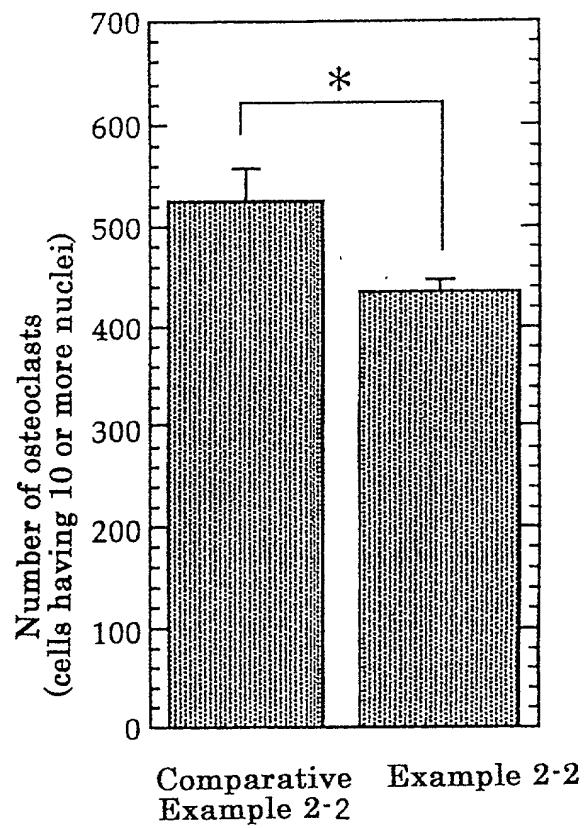
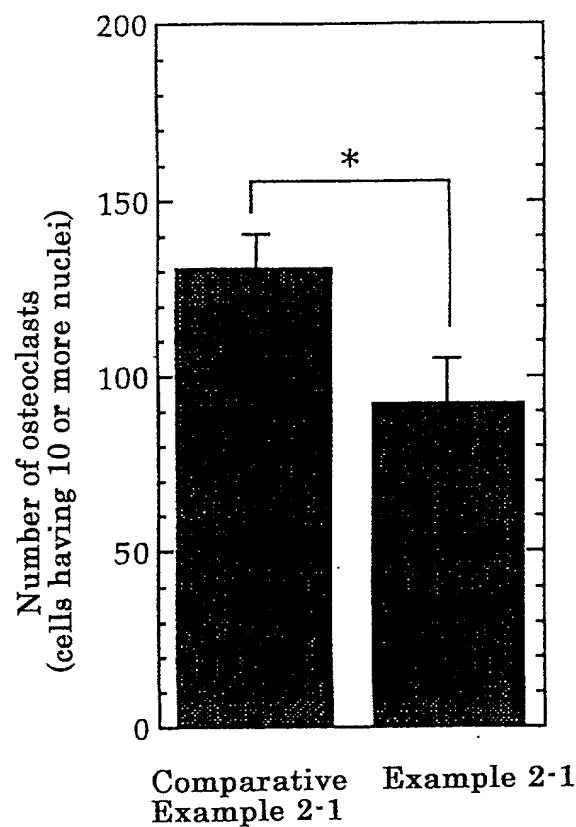


Figure 3

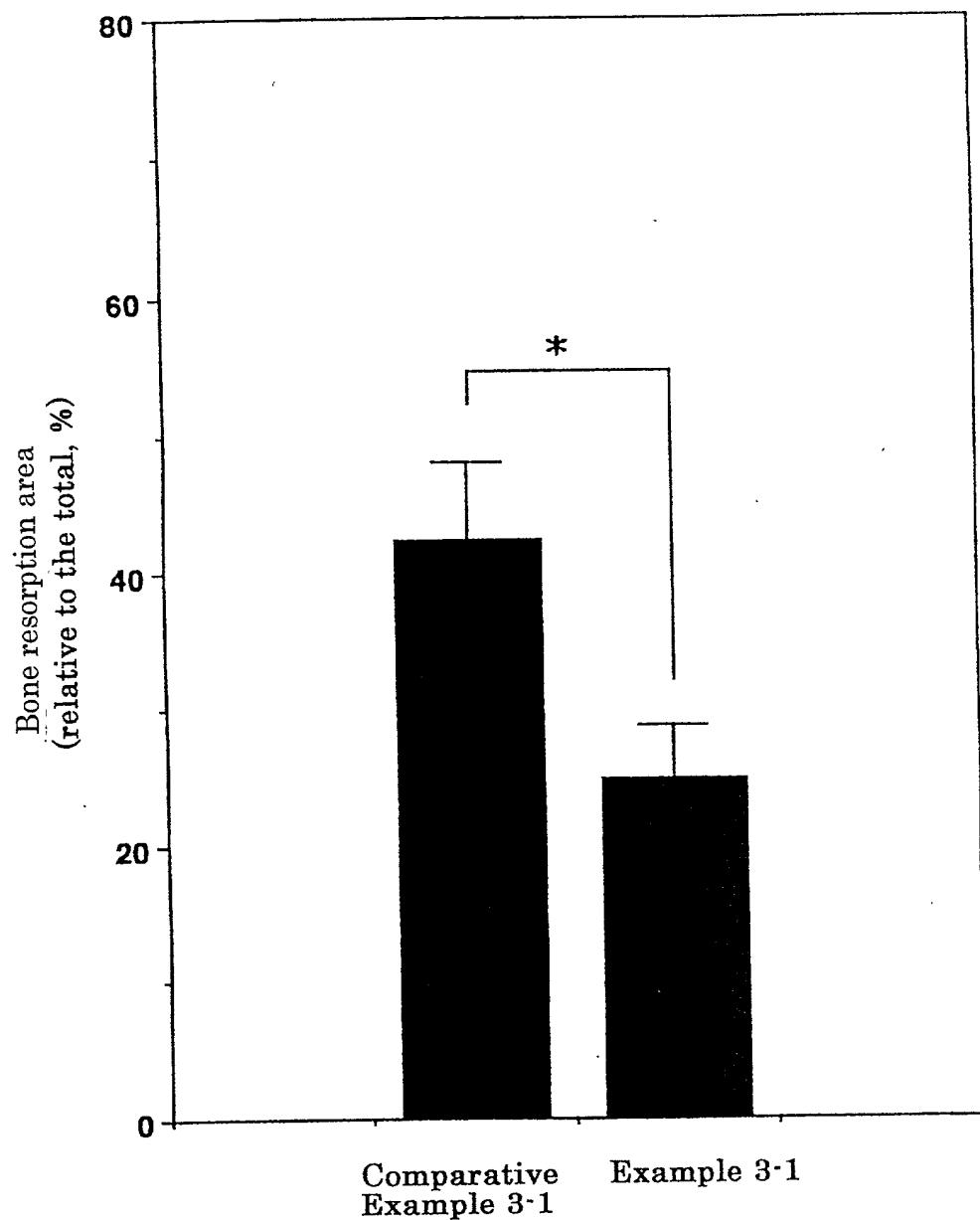
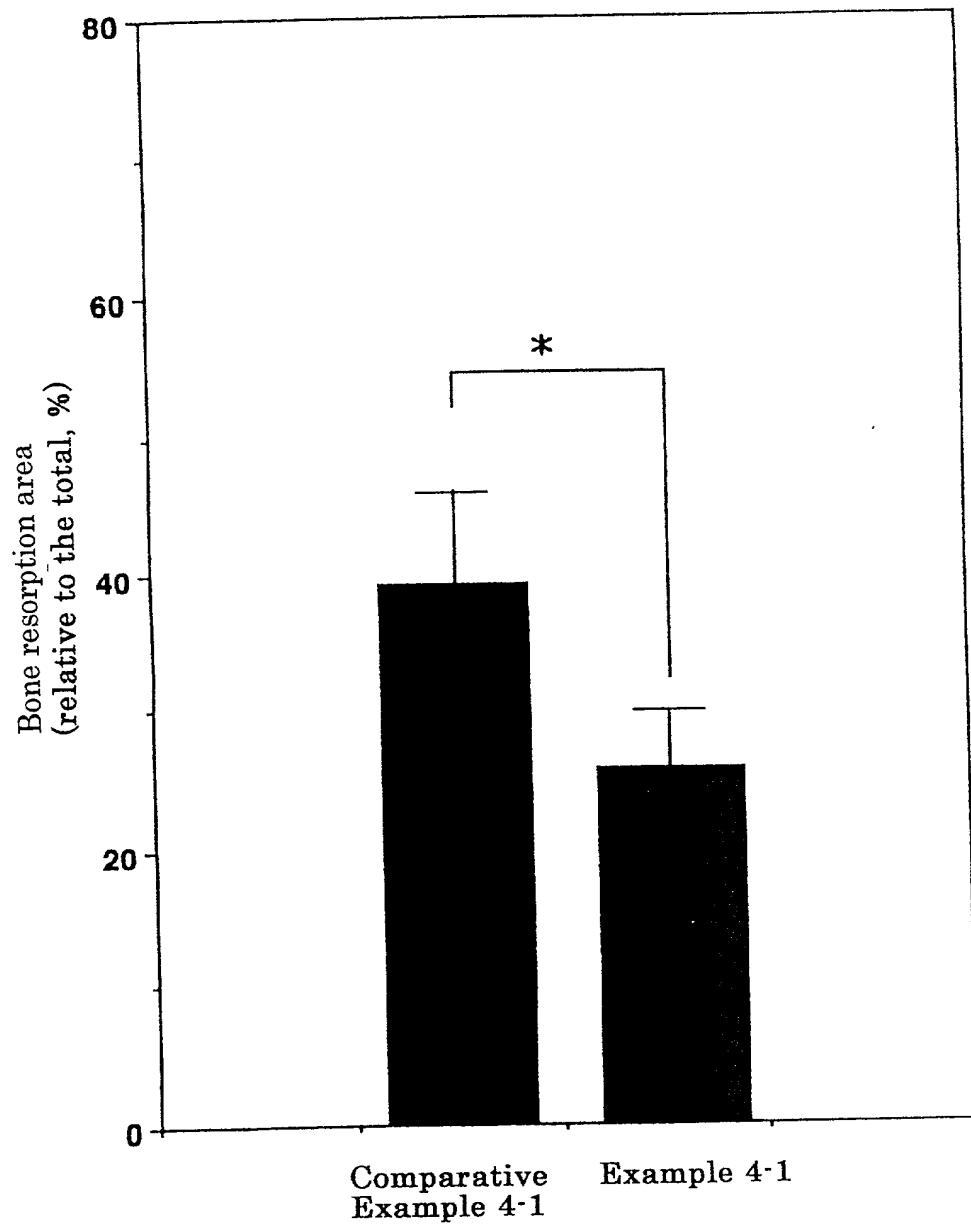


Figure 4



## Declaration and Power of Attorney For Patent Application

## 特許出願宣言書及び委任状

## Japanese Language Declaration

## 日本語宣言書

下記の氏名の発明者として、私は以下の通り宣言します。 As a below named inventor, I hereby declare that:

私の住所、私書箱、国籍は下記の私の氏名の後に記載された通りです。 My residence, post office address and citizenship are as stated next to my name.

下記の名称の発明について請求範囲に記載され、特許出願している発明内容について、私が最初かつ唯一の発明者（下記の氏名が一つの場合）もしくは最初かつ共同発明者であると（下記の名称が複数の場合）信じています。 I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

"METHODS FOR INHIBITING OSTEOCLAST FORMATION

OR METHODS FOR INHIBITING BONE RESORPTION"

上記発明の明細書（下記の欄で x 印がついていない場合は、the specification of which is attached hereto unless the following 本書に添付）は、

\_\_\_\_月\_\_\_\_日に提出され、米国出願番号または特許協定条約 国際出願番号を \_\_\_\_\_ とし、  
(該当する場合) \_\_\_\_\_ に訂正されました。  was filed on June 23, 2000  
as United States Application Number or  
PCT International Application Number  
PCT/JP00/04146 and was amended on  
\_\_\_\_\_ (if applicable).

私は、特許請求範囲を含む上記訂正後の明細書を検討し、内容を理解していることをここに表明します。

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

私は、連邦規則法典第37編第1条56項に定義されるとおり、特許資格の有無について重要な情報を開示する義務があることを認めます。

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

## Japanese Language Declaration (日本語宣言書)

私は、米国法典第35編119条(a)-(d)項又は365条(b)項に基づき下記の、米国以外の国の少なくとも一ヵ国を指定している特許協力条約365(a)項に基づく国際出願、又は外国での特許出願もしくは発明者証の出願について外国優先権をここに主張するとともに、優先権を主張している、本出願の前に出願された特許または発明者証の外国出願を以下に、枠内をマークすることで、示しています。

## Prior Foreign Application(s)

外国での先行出願

|                               |                            |   |   |  |
|-------------------------------|----------------------------|---|---|--|
| 11-284075<br>(Number)<br>(番号) | Japan<br>(Country)<br>(国名) | 5/October/1999<br>(Day/Month/Year Filed)<br>(出願年月日) | <input checked="" type="checkbox"/> Yes<br>はい | <input type="checkbox"/> No<br>いいえ     |
| <br>(Number)<br>(番号)          | <br>(Country)<br>(国名)      | <br>(Day/Month/Year Filed)<br>(出願年月日)               | <br><input type="checkbox"/> Yes<br>はい        | <br><input type="checkbox"/> No<br>いいえ |

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|---------------------------------|----------------------------|

|                                 |                            |
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私は、私自身の知識に基づいて本宣言書中で私が行なう表明が真実であり、かつ私の入手した情報と私の信じるところに基づく表明が全て真実であると信じていること、さらに故意になされた虚偽の表明及びそれと同等の行為は米国法典第18編第1001条に基づき、罰金または拘禁、もしくはその両方により処罰されること、そしてそのような故意による虚偽の声明を行なえば、出願した、又は既に許可された特許の有効性が失われることを認識し、よってここに上記のごとく宣言を致します。

I hereby claim foreign priority under Title 35, United States Code, Section 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Priority Claimed  
優先権主張

|   |  |
|---|--|
| <input checked="" type="checkbox"/> Yes<br>はい | <input type="checkbox"/> No<br>いいえ     |
| <br><input type="checkbox"/> Yes<br>はい        | <br><input type="checkbox"/> No<br>いいえ |

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I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s), or 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of application.

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| <br>(Status: Patented, Pending, Abandoned)<br>(現況:特許許可済、係属中、放棄済) |
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| <br>(Status: Patented, Pending, Abandoned)<br>(現況:特許許可済、係属中、放棄済) |
|--|

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

## Japanese Language Declaration (日本語宣言書)

委任状： 私は下記の発明者として、本出願に関する一切の手続きを米特許商標局に対して遂行する弁理士または代理人として、下記の者を指名いたします。（弁護士、または代理人の氏名及び登録番号を明記のこと）

|                         |        |
|-------------------------|--------|
| John E. McGarry         | 22,360 |
| H. Lawrence Smith       | 24,900 |
| Ralph T. Rader          | 28,772 |
| Joseph V. Coppola, Sr.  | 33,373 |
| Michael B. Stewart      | 36,018 |
| Alexander D. Rabinovich | 37,425 |
| Kevin D. Rutherford     | 40,412 |
| Glenn E. Forbis         | 40,610 |
| Ronald P. Kananen       | 24,104 |
| Matthew J. Russo        | 41,282 |
| G. Thomas Williams      | 42,228 |

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith (list name and registration number).

|                       |        |
|-----------------------|--------|
| Joel E. Bair          | 33,356 |
| Richard D. Grauer     | 22,388 |
| Michael D. Fishman    | 31,951 |
| Mark A. Davis         | 37,118 |
| Stefan V. Chmielewski | 39,914 |
| Shumel Livnat         | 33,949 |
| Kristin L. Murphy     | 41,212 |
| David K. Benson       | 42,314 |
| Christopher M. Tanner | 41,518 |
| Robert S. Green       | 41,800 |

## 書類送付先

RADER, FISHMAN & GRAUER PLLC  
1233 20th Street, N.W.  
Suite 501  
Washington, D.C. 20036

## Send Correspondence to:

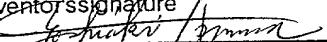
RADER, FISHMAN & GRAUER PLLC  
1233 20th Street, N.W.  
Suite 501  
Washington, D.C. 20036

## 直接電話連絡先： (名前及び電話番号)

(202) 955-3750

## Direct Telephone Calls to: (name and telephone number)

(202) 955-3750

|             |  |   |                             |
|-------------|--|---|-----------------------------|
| 唯一または第一発明者名 |  | Full name of sole or first inventor<br><u>Yoshiaki AZUMA</u>  |                             |
| 発明者署名       | 日付   | Inventory's signature<br>       | Date<br><u>May 14, 2001</u> |
| 住所          | Residence<br>TOKYO, JAPAN               |   |                             |
| 国籍          | Citizenship<br>Japanese  |   |                             |
| 私書箱         | Post Office Address c/o Teijin Limited, Tokyo<br>Research Center, 3-2, Asahigaoka 4-chome,<br>Hino-shi, Tokyo 191-0065 Japan |   |                             |
| 第二共同発明者     | Full name of second joint inventor, if any<br><u>Tomohiro OHTA</u>   |   |                             |
| 第二共同発明者署名   | 日付   | Second inventor's signature<br> | Date<br><u>May 16, 2001</u> |
| 住所          | Residence<br>TOKYO, JAPAN               |   |                             |
| 国籍          | Citizenship<br>Japanese  |   |                             |
| 私書箱         | Post Office Address c/o Teijin Limited, 1-1,<br>Uchisaiwaicho 2-chome, Chiyoda-ku,<br>Tokyo 100-0011 Japan                   |   |                             |

(第三以降の共同発明者についても同様に記載し、署名をすること)  
(Supply similar information and signature for third and subsequent joint inventors.)

Japanese Language Declaration

|                          |   |
|--------------------------|---|
| 第三共同発明者氏名<br><u>3-oo</u> | Full name of third joint inventor<br>Keiji KOMORIYA                           |
| 同発明者の署名<br>日付            | Inventor's signature<br><i>Keiji Komoriya</i> Date <u>May 18, 2001</u>        |
| 住所                       | Residence<br>TOKYO, JAPAN <u>JPX</u>  |
| 国籍                       | Citizenship<br>Japanese   |
| 郵便の宛先                    | Post Office Address<br>c/o Teijin Limited                                     |
|                          | 1-1, Uchisaiwaicho 2-chome, Chiyoda-ku,<br>Tokyo 100-0011 Japan               |
| 第四共同発明者<br><u>4-oo</u>   | Full name of fourth joint inventor, if any<br>Akira KUDO                      |
| 同発明者の署名<br>日付            | Inventor's signature<br><i>Akira Kudo</i> Date <u>May 12, 2001</u>            |
| 住所                       | Residence<br>TOKYO, JAPAN <u>JPX</u>  |
| 国籍                       | Citizenship<br>Japanese   |
| 郵便の宛先                    | Post Office Address<br>1-15-20, Nishi, Kunitachi-shi, Tokyo<br>186-0005 Japan |

|                          |  |
|--------------------------|--|
| 第五共同発明者氏名<br><u>5-oo</u> | Full name of fifth joint inventor<br>Sunao TAKESHITA   |
| 同発明者の署名<br>日付            | Inventor's signature<br><i>Sunao Takeshita</i> Date <u>May 12, 2001</u>  |
| 住所                       | Residence<br>KANAGAWA, JAPAN <u>JPX</u>  |
| 国籍                       | Citizenship<br>Japanese  |
| 郵便の宛先                    | Post Office Address<br>2-904, Lions Mansion Tsukimino, 2129-3,<br>Shimotsuruma, Yamato-shi, Kanagawa 242-0001<br>Japan |
| 第六共同発明者<br><u>6-oo</u>   | Full name of sixth joint inventor, if any<br>Keisuke KAJI  |
| 同発明者の署名<br>日付            | Inventor's signature<br><i>Keisuke Kaji</i> Date <u>May 12, 2001</u>   |
| 住所                       | Residence<br>KANAGAWA, JAPAN <u>JPX</u>  |
| 国籍                       | Citizenship<br>Japanese  |
| 郵便の宛先                    | Post Office Address<br>108, Hodogaya Bldg., 4-9-6, Nagatsuta,<br>Midori-ku, Yokohama-shi, Kanagawa 226-0027<br>Japan   |